

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark
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in its capacity as elected Office

Date of mailing (day/month/year) 11 September 2000 (11.09.00)	Applicant's or agent's file reference BB1262 PCT
International application No. PCT/US99/28586	Priority date (day/month/year) 03 December 1998 (03.12.98)
International filing date (day/month/year) 02 December 1999 (02.12.99)	
Applicant CAHOON, Rebecca, E. et al	

1. The designated Office is hereby notified of its election made:

☒ in the demand filed with the International Preliminary Examining Authority on:
26 June 2000 (26.06.00)

☐ in a notice effecting later election filed with the International Bureau on:

2. The election ☒ was
☐ was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No.: (41-22) 740.14.35	Authorized officer Athina Nickitas-Etienne Telephone No.: (41-22) 338.83.38
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PATENT COOPERATION TREATY

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INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference BB1262 PCT	FOR FURTHER ACTION see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. PCT/US 99/ 28586	International filing date (day/month/year) 02/12/1999	(Earliest) Priority Date (day/month/year) 08/12/1998
Applicant E. I DU PONT DE NEMOURS AND COMPANY ET AL		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.



It is also accompanied by a copy of each prior art document cited in this report.

1. Basis of the report

- a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.



the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

- b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :



contained in the international application in written form.



filed together with the international application in computer readable form.



furnished subsequently to this Authority in written form.



furnished subsequently to this Authority in computer readable form.



the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.



the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. ☐ **Certain claims were found unsearchable** (See Box I).

3. ☐ **Unity of invention is lacking** (see Box II).

4. With regard to the **title**,



the text is approved as submitted by the applicant.



the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,



the text is approved as submitted by the applicant.



the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No.



as suggested by the applicant.



because the applicant failed to suggest a figure.



because this figure better characterizes the invention.

1



None of the figures.



INTERNATIONAL SEARCH REPORT

International Application No.

US 99/28586

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/54 C12N15/82 C12N9/10 C12Q1/02 C12Q1/48
C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SASAKI T. ET AL.: "Rice EST AC D46513" EBI DATABASE, 9 March 1995 (1995-03-09), XP002139389 the whole document	1-9
X	SHINN P. ET AL.: "Genomic Arabidopsis sequence AC AC004557; Q9ZW42" EBI DATABASE, 15 April 1998 (1998-04-15), XP002139390 the whole document	1-10, 16



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

° Special categories of cited documents :

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Date of the actual completion of the international search

5 June 2000

Date of mailing of the international search report

26/06/2000

Name and mailing address of the ISA

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INTERNATIONAL SEARCH REPORT

International Application No

/US 99/28586

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WARDEN C. ET AL.: "Tissue-specific expression, developmental regulation, and chromosomal mapping of the lecithin:cholesterol acyltransferase gene" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 264, 1989, pages 21573-21581, XP002139391 the whole document ---	1-18
A	MURATA Y. ET AL.: "Cloning of rabbit LCAT cDNA: increase in LCAT mRNA abundance in the liver of cholesterol-fed rabbits" JOURNAL OF LIPID RESEARCH, vol. 37, 1996, pages 1616-1622, XP000913765 the whole document ---	1-18
A	WO 98 46767 A (TANIYAMA YOSHIO ; TAKEDA CHEMICAL INDUSTRIES LTD (JP)) 22 October 1998 (1998-10-22) see the whole document; esp. p.6 l.14 ff. ---	1-18
P,X	WALBOT V. ET AL.: "Maize EST, AC AW147120" EBI DATABASE, 4 November 1999 (1999-11-04), XP002139392 the whole document -----	1-9

Information on patent family members

US 99/28586

Form PCT/ISA/210 (patent family annex) (July 1992)



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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : C12N 15/54, 15/82, 9/10, C12Q 1/02, 1/48, 1/68		A2	(11) International Publication Number: WO 00/32791 (43) International Publication Date: 8 June 2000 (08.06.00)
(21) International Application Number: PCT/US99/28586 (22) International Filing Date: 2 December 1999 (02.12.99) (30) Priority Data: 60/110,782 3 December 1998 (03.12.98) US (71) Applicant (for all designated States except US): E.I. DU PONT DE NEMOURS AND COMPANY [US/US]; 1007 Market Street, Wilmington, DE 19898 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): CAHOON, Rebecca, E. [US/US]; 2331 West 18th Street, Wilmington, DE 19806 (US). KINNEY, Anthony, J. [GB/US]; 609 Lore Avenue, Wilmington, DE 19809 (US). SAKAI, Hajime [DE/US]; 105 Banbury Drive, Wilmington, DE 19803 (US). SHEN, Jennie, Bih-Jien [US/US]; 15 Bromley Court, Wilmington, DE 19810 (US). BUTLER, Karlene, H. [US/US]; 844 Broadfield Drive, Newark, DE 19713 (US). SAYLOR, James, J. [US/US]; 228 Duncan Avenue, Wilmington, DE 19803 (US). (74) Agent: FEULNER, Gregory, J.; E. I. du Pont de Nemours and Company, Legal Patent Records Center, 1007 Market Street, Wilmington, DE 19898 (US).			(81) Designated States: AE, AL, AU, BA, BB, BG, BR, CA, CN, CR, CU, CZ, DM, EE, GD, GE, HR, HU, ID, IL, IN, IS, JP, KP, KR, LC, LK, LR, LT, LV, MG, MK, MN, MX, NO, NZ, PL, RO, SG, SI, SK, SL, TR, TT, UA, US, UZ, VN, YU, ZA, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published Without international search report and to be republished upon receipt of that report.
(54) Title: PLAN LECITHIN:CHOLESTEROL ACYLTRANSFERASES			
(57) Abstract <p>This invention relates to an isolated nucleic acid fragment encoding a plant lecithin:cholesterol acyltransferases. The invention also relates to the construction of a chimeric gene encoding all or a portion of the plant lecithin:cholesterol acyltransferases, in sense or antisense orientation, wherein expression of the chimeric gene results in production of altered levels of the plant lecithin:cholesterol acyltransferases in a transformed host cell.</p>			
<p>Arabisidopsis (gi 3935185) MKK-----1-SSHYSVVIAILVVVTHSHCOVAGSN-----VYPLILVPGNGGNOLEVR corn_ (SEQ ID NO:2) HAR-1POVLAPLILLPLLAGLR--ELMIDRPLPKRCREVLHPLVLVPLCTCSELDAR corn_ (SEQ ID NO:4) MVNDMA-----CSRGGTIVLSKFASTTRAPKO-----LPPVVVPGYATNEDAR corn_ (SEQ ID NO:6) MASSLLOQLLSLLILLPLRLREHLSGNHVASAN-----NFWPFIAGVSCSDLEAR soybean_ (SEQ ID NO:8) MKKEQEGEL-KIEVATLTVTVVVVMSLLCTCGASN-----LDPLILPGNGGNOLEVR</p> <p>Arabisidopsis (gi 3935185) LDREYKPSVWNCSSMLYPINHKSGGFWRLWFDAAVLLSPFT-RCFSDRMHLYDDPDLDY corn_ (SEQ ID NO:2) LTDAYRPFRAACDE-----GGLVRLWTHNCSDPLPAHHYVRCFHEQMALVYDPVANDY corn_ (SEQ ID NO:4) LTLEYHPSSPRCA-----HKGKGFRLYINLYTALEDAADVRCFAEQMATAYDAASDDY corn_ (SEQ ID NO:6) LTLEYRPSVPHCGA-----HKGKGFRLWKNSSSELLSRDYVQCFAEQMSLVYDPAINEY soybean_ (SEQ ID NO:8) LTNOYKPSFTICESN-YPLIKKNGFWRLWFDSSVILAPFT-QCAERMTLHYHQELDDY</p> <p>Arabisidopsis (gi 3935185) ONAPGVOTRVPVPHFGSTKSLLYLDPLRLDATSYMEHLVKALEKKCCYVNDOTIL corn_ (SEQ ID NO:2) RNLPGVETRVNFGSSRGF-OKNPEHTTHSHCFEVLNRELARA-GYRDCOTLF corn_ (SEQ ID NO:4) RNAQGVETRVVFFGSTRAFRYDDPDRRTF-SYMDKTVSLRL-AYRDCENLF corn_ (SEQ ID NO:6) RNLACVETRVNFGSTRAFSHKNPLKSD--WCLGKLRAALDEM-GYRDCOTMF soybean_ (SEQ ID NO:8) FNTPGVETRVNFGSTNSLLYLNLPLKHTGYMPLVDSLOKL-GYADGETLF</p> <p>Arabisidopsis (gi 3935185) GLAASGHPSPRVASQFLQDLKQVLEKTSSENEGKPVILLS LFVHLFLMRTTPSWR corn_ (SEQ ID NO:2) APPVPGQPSRSSPATSVGVPSLVEDASRKNRGRKVLFG NVALEFVRSTPNAMR corn_ (SEQ ID NO:4) AVAPPGHSPRVADAFGLRLRLVERASRANGGGPVITVA TVAHQFLRLRPLPWR corn_ (SEQ ID NO:6) APPSPGOTSEVYSRYTKELMELVEAASERTR-KKAVILG NVALEFVRNTPPAMR soybean_ (SEQ ID NO:8) GLAAEGHPSQVGSFKLKLKLNLEEDASNSNNGKPVILLS LFVLOLLNRNPPSWR</p> <p>Arabisidopsis (gi 3935185) RKYIKHFLVALAAPH-GGTISONKTFASGNTLG-VPLVNLPL--LVRRKQRTSESQNWLLPS corn_ (SEQ ID NO:2) DRYIKHFLVALAAPHGAGFVPLQYFVSGSNLHYVPTVSSLEPAFRPHMRTFESSLVNFP corn_ (SEQ ID NO:4) RRFVRFPVVAAPH-GGVVLGHLTIAGNHLG-LFFVDPL--ALKEYSRSLQSSWLPN corn_ (SEQ ID NO:6) REHIERLVVAPTLPCGFLEFVRNFGSTDLIYVATTPLAT--RAHWRSTESAIWNFP soybean_ (SEQ ID NO:8) KKKIKHFLIALSAPH-GGAIDENYTFASGNTLG-VPLVNLPL--LVRRKQRTSESQNWLLPS</p> <p>Arabisidopsis (gi 3935185) TKVFHDKTKPLVTPOVNYTA--YENDRFADIGFSGGVVYKTVLPLTEELMTPCGV corn_ (SEQ ID NO:2) PAVTGR--RPLVVTARRNYSA--YDLELLVAVGCGAGVEPTRRRAVPHKSYGAPNVP corn_ (SEQ ID NO:4) PNAFRA--GQPLVTTASRTYTA--NDMADFLDAIGLGAIVFYQSRVPLFRLESPRPV corn_ (SEQ ID NO:6) PAVTGRGQPLVVTRENRYSASANDMERFLAAVGSGEAAEPTRRRAVPHKSGFAAPNVP soybean_ (SEQ ID NO:8) PKITF-GPOKPIVITPIRPSYA--NDMVDLKDIGFPEGVYPTETRLPLIGNIKAPQVFI</p> <p>Arabisidopsis (gi 3935185) TCYI KOPEIK ASLAAAL-----KVDS--LN corn_ (SEQ ID NO:2) TCNN ATPEIV VSMIAFDEKMRROPEQNKVYK corn_ (SEQ ID NO:4) ACVV VTPMNV VELLAVDP-AKRLPTA-Y-TR corn_ (SEQ ID NO:6) TYIS AAPEVAA ISVLAFEKMRROPEQNKQK soybean_ (SEQ ID NO:8) TCIN ERPEIS VSLAALQS-LMKKKNQY-LK</p> <p>Arabisidopsis (gi 3935185) TVEIDGVSHTSILKDEIALKEIMKQISIINY--ELANVNVANE corn_ (SEQ ID NO:2) SIKIRGAQHTGIVTDDTALKRVHHEILEANR-----S corn_ (SEQ ID NO:4) HLKVRNVSHNTGLFVDDAALAVIISAT-----LRPN corn_ (SEQ ID NO:6) SIKINKAQHTIVTDDFALNRVIGIIVEANNQK-----IPS soybean_ (SEQ ID NO:8) VVKIDGVSHTSILKDEIALKEIMKQISIINY--ELANVNVANE</p>			

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TITLE

PLANT LECITHIN:CHOLESTEROL ACYLTRANSFERASES

This application claims the benefit of U.S. Provisional Application No. 60/110,782, filed December 3, 1998.

FIELD OF THE INVENTION

This invention is in the field of plant molecular biology. More specifically, this invention pertains to nucleic acid fragments encoding plant lecithin:cholesterol acyltransferases in plants and seeds.

BACKGROUND OF THE INVENTION

Phosphatidylcholine-sterol O-acyltransferase (EC 2.3.1.43) transfers acyl groups from phosphatidylcholine to sterols. This enzyme is also known as lecithin:cholesterol acyltransferase (LCAT) and belongs to the class of CoA-independent acyltransferases. This enzyme is found associated with high-density lipoproteins (HDL) and forming a complex with its activators Apolipoprotein (apo)-A1 and apo-D. HDLs are believed to promote the process of reverse cholesterol transport. This process involves efflux of cellular cholesterol, cholesterol esterification and lipid transport and exchange. Apo A-I and lecithin-cholesterol acyltransferase play a crucial role in reverse cholesterol transport.

The role of LCAT in plants will presumably be different from mammalian systems given the negligible levels of cholesterol found in plant oils. However, plants have a complex combination of membrane sterols that can change with environmental conditions as well as developmental determination. LCAT may function as the phosphatidylcholine acyl-exchange enzyme which moves unsaturated acyl groups into phosphatidylcholine for desaturation and out of it for incorporation into triacylglycerols. Overexpression of LCAT may lead to increased lipid metabolism and fluidity of membranes increasing resistance to heat and/or cold shock. Overexpression or cosuppression of LCAT may also be useful to genetically alter the content of phytosterol or lecithin in grains.

SUMMARY OF THE INVENTION

The present invention relates to isolated polynucleotides comprising a nucleotide sequence encoding a first polypeptide of at least 417 amino acids that has at least 60% identity based on the Clustal method of alignment when compared to a polypeptide selected from the group consisting of a corn plant lecithin:cholesterol acyltransferases polypeptide of SEQ ID NOs:2, 4, 8, 10, or 12, and a soybean plant lecithin:cholesterol acyltransferases polypeptide of SEQ ID NOs:6, or 14. The present invention also relates to an isolated polynucleotide comprising the complement of the nucleotide sequences described above.

It is preferred that the isolated polynucleotides of the claimed invention consists of a nucleic acid sequence selected from the group consisting of SEQ ID NOs:1, 3, 5, 7, 9, 11, and 13 that codes for the polypeptide selected from the group consisting of SEQ ID NOs:2, 4, 6, 8, 10, 12, and 14. The present invention also relates to an isolated polynucleotide

comprising a nucleotide sequences of at least one of 60 (preferably at least one of 40, most preferably at least one of 30) contiguous nucleotides derived from a nucleotide sequence selected from the group consisting of SEQ ID NOs:1, 3, 5, 7, 9, 11, and 13 and the complement of such nucleotide sequences.

5 The present invention relates to an isolated polynucleotide comprising at least one of 30 contiguous nucleotides derived from a nucleotide sequence selected from the group consisting of SEQ ID NOs:1, 3, 5, 7, 9, 11, 13 and the complement of such sequences.

The present invention relates to a chimeric gene comprising an isolated polynucleotide of the present invention operably linked to suitable regulatory sequences.

10 The present invention relates to an isolated host cell comprising a chimeric gene of the present invention or an isolated polynucleotide of the present invention. The host cell may be eukaryotic, such as a yeast or a plant cell, or prokaryotic, such as a bacterial cell. The present invention also relates to a virus, preferably a baculovirus, comprising an isolated polynucleotide of the present invention or a chimeric gene of the present invention.

15 The present invention relates to a process for producing an isolated host cell comprising a chimeric gene of the present invention or an isolated polynucleotide of the present invention, the process comprising either transforming or transfecting an isolated compatible host cell with a chimeric gene or isolated polynucleotide of the present invention.

20 The present invention relates to a plant lecithin:cholesterol acyltransferases polypeptide of at least 417 amino acids comprising at least 60% homology based on the Clustal method of alignment compared to a polypeptide selected from the group consisting of SEQ ID NOs:2, 4, 6, 8, 10, 12, and 14.

25 The present invention relates to a method of selecting an isolated polynucleotide that affects the level of expression of a plant lecithin:cholesterol acyltransferases polypeptide in a host cell, preferably a plant cell, the method comprising the steps of:

constructing an isolated polynucleotide of the present invention or an isolated chimeric gene of the present invention;

introducing the isolated polynucleotide or the isolated chimeric gene into a host cell;

30 measuring the level a plant lecithin:cholesterol acyltransferases polypeptide in the host cell containing the isolated polynucleotide; and

35 comparing the level of a plant lecithin:cholesterol acyltransferases polypeptide in the host cell containing the isolated polynucleotide with the level of a plant lecithin:cholesterol acyltransferases polypeptide in a host cell that does not contain the isolated polynucleotide.

The present invention relates to a method of obtaining a nucleic acid fragment encoding a substantial portion of a lecithin:cholesterol acyltransferases polypeptide gene, preferably a plant plant lecithin:cholesterol acyltransferases polypeptide gene, comprising

the steps of: synthesizing an oligonucleotide primer comprising a nucleotide sequence of at least one of 60 (preferably at least one of 40, most preferably at least one of 30) contiguous nucleotides derived from a nucleotide sequence selected from the group consisting of SEQ ID NOs:1, 3, 5, 7, 9, 11, and 13 and the complement of such nucleotide sequences; and
5 amplifying a nucleic acid fragment (preferably a cDNA inserted in a cloning vector) using the oligonucleotide primer. The amplified nucleic acid fragment preferably will encode a portion of a plant lecithin:cholesterol acyltransferases amino acid sequence.

The present invention also relates to a method of obtaining a nucleic acid fragment encoding all or a substantial portion of the amino acid sequence encoding a plant
10 lecithin:cholesterol acyltransferases polypeptide comprising the steps of: probing a cDNA or genomic library with an isolated polynucleotide of the present invention; identifying a DNA clone that hybridizes with an isolated polynucleotide of the present invention; isolating the identified DNA clone; and sequencing the cDNA or genomic fragment that comprises the isolated DNA clone.

A further embodiment of the instant invention is a method for evaluating at least one compound for its ability to inhibit the activity of a plant lecithin:cholesterol acyltransferases, the method comprising the steps of: (a) transforming a host cell with a chimeric gene comprising a nucleic acid fragment encoding a plant lecithin:cholesterol acyltransferases, operably linked to suitable regulatory sequences; (b) growing the transformed host cell under
20 conditions that are suitable for expression of the chimeric gene wherein expression of the chimeric gene results in production of plant lecithin:cholesterol acyltransferases in the transformed host cell; (c) optionally purifying the plant lecithin:cholesterol acyltransferases expressed by the transformed host cell; (d) treating the plant lecithin:cholesterol acyltransferases with a compound to be tested; and (e) comparing the activity of the plant
25 lecithin:cholesterol acyltransferases that has been treated with a test compound to the activity of an untreated plant lecithin:cholesterol acyltransferases, thereby selecting compounds with potential for inhibitory activity.

BRIEF DESCRIPTION OF THE DRAWINGS AND SEQUENCE DESCRIPTIONS

30 The invention can be more fully understood from the following detailed description and the accompanying drawings and Sequence Listing which form a part of this application.

Figure 1 depicts the amino acid sequence alignment between the lecithin:cholesterol acyltransferase corn clone cen3n.pk0141.f2:fis (SEQ ID NO:8), corn clone cep1c.pk001.f7:fis (SEQ ID NO:10), corn clone chpc24.pk0001.c1 (SEQ ID NO:12), and
35 soybean clone sl2.pk0015.e8:fis (SEQ ID NO:14), with the *Arabidopsis thaliana* lecithin:cholesterol acyltransferase protein (NCBI General Identifier No. 3935185; SEQ ID NO:15). Conserved sequence elements are shown boxed in black with the amino acid sequence in white. The second boxed sequence (HS*G) contains a conserved serine that is

believed to be the active site residue found in all serine lipases. This sequence motif is also conserved in mammalian lecithin:cholesterol acyltransferases (Rogne et al. (1987) *Biochem Biophys Res Commun* 148:161-169). All of the boxed sequences are largely conserved in the mammalian lecithin:cholesterol acyltransferase sequences.

5 Table 1 lists the polypeptides that are described herein, the designation of the cDNA clones that comprise the nucleic acid fragments encoding polypeptides representing all or a substantial portion of these polypeptides, and the corresponding identifier (SEQ ID NO:) as used in the attached Sequence Listing. The sequence descriptions and Sequence Listing attached hereto comply with the rules governing nucleotide and/or amino acid sequence
10 disclosures in patent applications as set forth in 37 C.F.R. §1.821-1.825.

TABLE 1
Lecithin:Cholesterol Acyltransferases

Lecithin:Cholesterol Acyltransferase (LCAT)	Clone Designation	SEQ ID NO:	
		(Nucleotide)	(Amino Acid)
maize [Zea mays]	cep1c.pk001.f7	1	2
maize [Zea mays]	Contig of: chpc24.pk0001.c1 cbn2.pk0017.h4 cbn10.pk0052.g10 p0016.ctsbo30r p0018.chstj36r	3	4
soybean [Glycine max]	sl2.pk0015.e8	5	6
maize [Zea mays]	cen3n.pk0141.f2:fis	7	8
maize [Zea mays]	cep1c.pk001.f7:fis	9	10
maize [Zea mays]	chpc24.pk0001.c1	11	12
soybean [Glycine max]	sl2.pk0015.e8:fis	13	14

15 The Sequence Listing contains the one letter code for nucleotide sequence characters and the three letter codes for amino acids as defined in conformity with the IUPAC-IUBMB standards described in *Nucleic Acids Res.* 13:3021-3030 (1985) and in the *Biochemical J.* 219 (No. 2):345-373 (1984) which are herein incorporated by reference. The symbols and
20 format used for nucleotide and amino acid sequence data comply with the rules set forth in 37 C.F.R. §1.822.

DETAILED DESCRIPTION OF THE INVENTION

In the context of this disclosure, a number of terms shall be utilized. As used herein, a "polynucleotide" is a nucleotide sequence such as a nucleic acid fragment. A polynucleotide may be a polymer of RNA or DNA that is single- or double-stranded, that optionally
25 contains synthetic, non-natural or altered nucleotide bases. A polynucleotide in the form of a polymer of DNA may be comprised of one or more segments of cDNA, genomic DNA,

synthetic DNA, or mixtures thereof. An isolated polynucleotide of the present invention may include at least one of 60 contiguous nucleotides, preferably at least one of 40 contiguous nucleotides, most preferably one of at least 30 contiguous nucleotides, of the nucleic acid sequence of the SEQ ID NOs:1, 3, 5, 7, 9, 11, 13, or the complement of such sequences.

As used herein, "contig" refers to a nucleotide sequence that is assembled from two or more constituent nucleotide sequences that share common or overlapping regions of sequence homology. For example, the nucleotide sequences of two or more nucleic acid fragments can be compared and aligned in order to identify common or overlapping sequences. Where common or overlapping sequences exist between two or more nucleic acid fragments, the sequences (and thus their corresponding nucleic acid fragments) can be assembled into a single contiguous nucleotide sequence.

As used herein, "substantially similar" refers to nucleic acid fragments wherein changes in one or more nucleotide bases results in substitution of one or more amino acids, but do not affect the functional properties of the polypeptide encoded by the nucleotide sequence. "Substantially similar" also refers to nucleic acid fragments wherein changes in one or more nucleotide bases does not affect the ability of the nucleic acid fragment to mediate alteration of gene expression by gene silencing through for example antisense or co-suppression technology. "Substantially similar" also refers to modifications of the nucleic acid fragments of the instant invention such as deletion or insertion of one or more nucleotides that do not substantially affect the functional properties of the resulting transcript vis-à-vis the ability to mediate gene silencing or alteration of the functional properties of the resulting protein molecule. It is therefore understood that the invention encompasses more than the specific exemplary nucleotide or amino acid sequences and includes functional equivalents thereof.

Substantially similar nucleic acid fragments may be selected by screening nucleic acid fragments representing subfragments or modifications of the nucleic acid fragments of the instant invention, wherein one or more nucleotides are substituted, deleted and/or inserted, for their ability to affect the level of the polypeptide encoded by the unmodified nucleic acid fragment in a plant or plant cell. For example, a substantially similar nucleic acid fragment representing at least one of 30 contiguous nucleotides derived from the instant nucleic acid fragment can be constructed and introduced into a plant or plant cell. The level of the polypeptide encoded by the unmodified nucleic acid fragment present in a plant or plant cell exposed to the substantially similar nucleic fragment can then be compared to the level of the polypeptide in a plant or plant cell that is not exposed to the substantially similar nucleic acid fragment.

For example, it is well known in the art that antisense suppression and co-suppression of gene expression may be accomplished using nucleic acid fragments representing less than

the entire coding region of a gene, and by nucleic acid fragments that do not share 100% sequence identity with the gene to be suppressed. Moreover, alterations in a nucleic acid fragment which result in the production of a chemically equivalent amino acid at a given site, but do not effect the functional properties of the encoded polypeptide, are well known in the art. Thus, a codon for the amino acid alanine, a hydrophobic amino acid, may be substituted by a codon encoding another less hydrophobic residue, such as glycine, or a more hydrophobic residue, such as valine, leucine, or isoleucine. Similarly, changes which result in substitution of one negatively charged residue for another, such as aspartic acid for glutamic acid, or one positively charged residue for another, such as lysine for arginine, can also be expected to produce a functionally equivalent product. Nucleotide changes which result in alteration of the N-terminal and C-terminal portions of the polypeptide molecule would also not be expected to alter the activity of the polypeptide. Each of the proposed modifications is well within the routine skill in the art, as is determination of retention of biological activity of the encoded products. Consequently, an isolated polynucleotide comprising a nucleotide sequence of at least one of 60 (preferably at least one of 40, most preferably at least one of 30) contiguous nucleotides derived from a nucleotide sequence selected from the group consisting of SEQ ID NOs:1, 3, 5, 7, 9, 11, 13 and the complement of such nucleotide sequences may be used in methods of selecting an isolated polynucleotide that affects the expression of a polypeptide (such as lecithin:cholesterol acyltransferases) in a host cell. A method of selecting an isolated polynucleotide that affects the level of expression of a polypeptide in a host cell (eukaryotic, such as plant or yeast, prokaryotic such as bacterial, or viral) may comprise the steps of: constructing an isolated polynucleotide of the present invention or an isolated chimeric gene of the present invention; introducing the isolated polynucleotide or the isolated chimeric gene into a host cell; measuring the level a polypeptide in the host cell containing the isolated polynucleotide; and comparing the level of a polypeptide in the host cell containing the isolated polynucleotide with the level of a polypeptide in a host cell that does not contain the isolated polynucleotide.

Moreover, substantially similar nucleic acid fragments may also be characterized by their ability to hybridize. Estimates of such homology are provided by either DNA-DNA or DNA-RNA hybridization under conditions of stringency as is well understood by those skilled in the art (Hames and Higgins, Eds. (1985) *Nucleic Acid Hybridisation*, IRL Press, Oxford, U.K.). Stringency conditions can be adjusted to screen for moderately similar fragments, such as homologous sequences from distantly related organisms, to highly similar fragments, such as genes that duplicate functional enzymes from closely related organisms. Post-hybridization washes determine stringency conditions. One set of preferred conditions uses a series of washes starting with 6X SSC, 0.5% SDS at room temperature for 15 min, then repeated with 2X SSC, 0.5% SDS at 45°C for 30 min, and then repeated twice with

0.2X SSC, 0.5% SDS at 50°C for 30 min. A more preferred set of stringent conditions uses higher temperatures in which the washes are identical to those above except for the temperature of the final two 30 min washes in 0.2X SSC, 0.5% SDS was increased to 60°C. Another preferred set of highly stringent conditions uses two final washes in 0.1X SSC,
5 0.1% SDS at 65°C.

Substantially similar nucleic acid fragments of the instant invention may also be characterized by the percent identity of the amino acid sequences that they encode to the amino acid sequences disclosed herein, as determined by algorithms commonly employed by those skilled in this art. Suitable nucleic acid fragments (isolated polynucleotides of the
10 present invention) encode polypeptides that are at least about 70% identical, preferably at least about 80% identical to the amino acid sequences reported herein. Preferred nucleic acid fragments encode amino acid sequences that are at least about 85% identical to the amino acid sequences reported herein. More preferred nucleic acid fragments encode amino acid sequences that are at least about 90% identical to the amino acid sequences reported
15 herein. Most preferred are nucleic acid fragments that encode amino acid sequences that are at least about 95% identical to the amino acid sequences reported herein. Suitable nucleic acid fragments not only have the above homologies but typically encode a polypeptide having at least about 50 amino acids, preferably at least about 100 amino acids, more preferably at least about 150 amino acids, still more preferably at least about 200 amino
20 acids, and most preferably at least about 250 amino acids. Sequence alignments and percent identity calculations were performed using the Megalign program of the LASERGENE bioinformatics computing suite (DNASTAR Inc., Madison, WI). Multiple alignment of the sequences was performed using the Clustal method of alignment (Higgins and Sharp (1989) *CABIOS*. 5:151-153) with the default parameters (GAP PENALTY=10, GAP LENGTH
25 PENALTY=10). Default parameters for pairwise alignments using the Clustal method were KTUPLE 1, GAP PENALTY=3, WINDOW=5 and DIAGONALS SAVED=5.

A "substantial portion" of an amino acid or nucleotide sequence comprises an amino acid or a nucleotide sequence that is sufficient to afford putative identification of the protein or gene that the amino acid or nucleotide sequence comprises. Amino acid and nucleotide
30 sequences can be evaluated either manually by one skilled in the art, or by using computer-based sequence comparison and identification tools that employ algorithms such as BLAST (Basic Local Alignment Search Tool; Altschul et al. (1993) *J. Mol. Biol.* 215:403-410; see also www.ncbi.nlm.nih.gov/BLAST/). In general, a sequence of ten or more contiguous amino acids or thirty or more contiguous nucleotides is necessary in order to putatively
35 identify a polypeptide or nucleic acid sequence as homologous to a known protein or gene. Moreover, with respect to nucleotide sequences, gene-specific oligonucleotide probes comprising 30 or more contiguous nucleotides may be used in sequence-dependent methods of gene identification (e.g., Southern hybridization) and isolation (e.g., *in situ* hybridization

of bacterial colonies or bacteriophage plaques). In addition, short oligonucleotides of 12 or more nucleotides may be used as amplification primers in PCR in order to obtain a particular nucleic acid fragment comprising the primers. Accordingly, a "substantial portion" of a nucleotide sequence comprises a nucleotide sequence that will afford specific identification and/or isolation of a nucleic acid fragment comprising the sequence. The instant specification teaches amino acid and nucleotide sequences encoding polypeptides that comprise one or more particular plant proteins. The skilled artisan, having the benefit of the sequences as reported herein, may now use all or a substantial portion of the disclosed sequences for purposes known to those skilled in this art. Accordingly, the instant invention comprises the complete sequences as reported in the accompanying Sequence Listing, as well as substantial portions of those sequences as defined above.

"Codon degeneracy" refers to divergence in the genetic code permitting variation of the nucleotide sequence without effecting the amino acid sequence of an encoded polypeptide. Accordingly, the instant invention relates to any nucleic acid fragment comprising a nucleotide sequence that encodes all or a substantial portion of the amino acid sequences set forth herein. The skilled artisan is well aware of the "codon-bias" exhibited by a specific host cell in usage of nucleotide codons to specify a given amino acid. Therefore, when synthesizing a nucleic acid fragment for improved expression in a host cell, it is desirable to design the nucleic acid fragment such that its frequency of codon usage approaches the frequency of preferred codon usage of the host cell.

"Synthetic nucleic acid fragments" can be assembled from oligonucleotide building blocks that are chemically synthesized using procedures known to those skilled in the art. These building blocks are ligated and annealed to form larger nucleic acid fragments which may then be enzymatically assembled to construct the entire desired nucleic acid fragment. "Chemically synthesized", as related to nucleic acid fragment, means that the component nucleotides were assembled *in vitro*. Manual chemical synthesis of nucleic acid fragments may be accomplished using well established procedures, or automated chemical synthesis can be performed using one of a number of commercially available machines. Accordingly, the nucleic acid fragments can be tailored for optimal gene expression based on optimization of nucleotide sequence to reflect the codon bias of the host cell. The skilled artisan appreciates the likelihood of successful gene expression if codon usage is biased towards those codons favored by the host. Determination of preferred codons can be based on a survey of genes derived from the host cell where sequence information is available.

"Gene" refers to a nucleic acid fragment that expresses a specific protein, including regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence. "Native gene" refers to a gene as found in nature with its own regulatory sequences. "Chimeric gene" refers any gene that is not a native gene, comprising regulatory and coding sequences that are not found together in nature.

Accordingly, a chimeric gene may comprise regulatory sequences and coding sequences that are derived from different sources, or regulatory sequences and coding sequences derived from the same source, but arranged in a manner different than that found in nature.

“Endogenous gene” refers to a native gene in its natural location in the genome of an organism. A “foreign” gene refers to a gene not normally found in the host organism, but that is introduced into the host organism by gene transfer. Foreign genes can comprise native genes inserted into a non-native organism, or chimeric genes. A “transgene” is a gene that has been introduced into the genome by a transformation procedure.

“Coding sequence” refers to a nucleotide sequence that codes for a specific amino acid sequence. “Regulatory sequences” refer to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences may include promoters, translation leader sequences, introns, and polyadenylation recognition sequences.

“Promoter” refers to a nucleotide sequence capable of controlling the expression of a coding sequence or functional RNA. In general, a coding sequence is located 3' to a promoter sequence. The promoter sequence consists of proximal and more distal upstream elements, the latter elements often referred to as enhancers. Accordingly, an “enhancer” is a nucleotide sequence which can stimulate promoter activity and may be an innate element of the promoter or a heterologous element inserted to enhance the level or tissue-specificity of a promoter. Promoters may be derived in their entirety from a native gene, or be composed of different elements derived from different promoters found in nature, or even comprise synthetic nucleotide segments. It is understood by those skilled in the art that different promoters may direct the expression of a gene in different tissues or cell types, or at different stages of development, or in response to different environmental conditions. Promoters which cause a nucleic acid fragment to be expressed in most cell types at most times are commonly referred to as “constitutive promoters”. New promoters of various types useful in plant cells are constantly being discovered; numerous examples may be found in the compilation by Okamuro and Goldberg (1989) *Biochemistry of Plants* 15:1-82. It is further recognized that since in most cases the exact boundaries of regulatory sequences have not been completely defined, nucleic acid fragments of different lengths may have identical promoter activity.

The “translation leader sequence” refers to a nucleotide sequence located between the promoter sequence of a gene and the coding sequence. The translation leader sequence is present in the fully processed mRNA upstream of the translation start sequence. The translation leader sequence may affect processing of the primary transcript to mRNA, mRNA stability or translation efficiency. Examples of translation leader sequences have been described (Turner and Foster (1995) *Mol. Biotechnol.* 3:225-236).

The "3' non-coding sequences" refer to nucleotide sequences located downstream of a coding sequence and include polyadenylation recognition sequences and other sequences encoding regulatory signals capable of affecting mRNA processing or gene expression. The polyadenylation signal is usually characterized by affecting the addition of polyadenylic acid tracts to the 3' end of the mRNA precursor. The use of different 3' non-coding sequences is exemplified by Ingelbrecht et al. (1989) *Plant Cell* 1:671-680.

"RNA transcript" refers to the product resulting from RNA polymerase-catalyzed transcription of a DNA sequence. When the RNA transcript is a perfect complementary copy of the DNA sequence, it is referred to as the primary transcript or it may be a RNA sequence derived from posttranscriptional processing of the primary transcript and is referred to as the mature RNA. "Messenger RNA (mRNA)" refers to the RNA that is without introns and that can be translated into polypeptide by the cell. "cDNA" refers to a double-stranded DNA that is complementary to and derived from mRNA. "Sense" RNA refers to an RNA transcript that includes the mRNA and so can be translated into a polypeptide by the cell. "Antisense RNA" refers to an RNA transcript that is complementary to all or part of a target primary transcript or mRNA and that blocks the expression of a target gene (see U.S. Patent No. 5,107,065, incorporated herein by reference). The complementarity of an antisense RNA may be with any part of the specific nucleotide sequence, i.e., at the 5' non-coding sequence, 3' non-coding sequence, introns, or the coding sequence. "Functional RNA" refers to sense RNA, antisense RNA, ribozyme RNA, or other RNA that may not be translated but yet has an effect on cellular processes.

The term "operably linked" refers to the association of two or more nucleic acid fragments on a single nucleic acid fragment so that the function of one is affected by the other. For example, a promoter is operably linked with a coding sequence when it is capable of affecting the expression of that coding sequence (i.e., that the coding sequence is under the transcriptional control of the promoter). Coding sequences can be operably linked to regulatory sequences in sense or antisense orientation.

The term "expression", as used herein, refers to the transcription and stable accumulation of sense (mRNA) or antisense RNA derived from the nucleic acid fragment of the invention. Expression may also refer to translation of mRNA into a polypeptide. "Antisense inhibition" refers to the production of antisense RNA transcripts capable of suppressing the expression of the target protein. "Overexpression" refers to the production of a gene product in transgenic organisms that exceeds levels of production in normal or non-transformed organisms. "Co-suppression" refers to the production of sense RNA transcripts capable of suppressing the expression of identical or substantially similar foreign or endogenous genes (U.S. Patent No. 5,231,020, incorporated herein by reference).

"Altered levels" refers to the production of gene product(s) in transgenic organisms in amounts or proportions that differ from that of normal or non-transformed organisms.

“Mature” protein refers to a post-translationally processed polypeptide; i.e., one from which any pre- or propeptides present in the primary translation product have been removed. “Precursor” protein refers to the primary product of translation of mRNA; i.e., with pre- and propeptides still present. Pre- and propeptides may be but are not limited to intracellular localization signals.

A “chloroplast transit peptide” is an amino acid sequence which is translated in conjunction with a protein and directs the protein to the chloroplast or other plastid types present in the cell in which the protein is made. “Chloroplast transit sequence” refers to a nucleotide sequence that encodes a chloroplast transit peptide. A “signal peptide” is an amino acid sequence which is translated in conjunction with a protein and directs the protein to the secretory system (Chrispeels (1991) *Ann. Rev. Plant Phys. Plant Mol. Biol.* 42:21-53). If the protein is to be directed to a vacuole, a vacuolar targeting signal (*supra*) can further be added, or if to the endoplasmic reticulum, an endoplasmic reticulum retention signal (*supra*) may be added. If the protein is to be directed to the nucleus, any signal peptide present should be removed and instead a nuclear localization signal included (Raikhel (1992) *Plant Phys.* 100:1627-1632).

“Transformation” refers to the transfer of a nucleic acid fragment into the genome of a host organism, resulting in genetically stable inheritance. Host organisms containing the transformed nucleic acid fragments are referred to as “transgenic” organisms. Examples of methods of plant transformation include *Agrobacterium*-mediated transformation (De Blaere et al. (1987) *Meth. Enzymol.* 143:277) and particle-accelerated or “gene gun” transformation technology (Klein et al. (1987) *Nature (London)* 327:70-73; U.S. Patent No. 4,945,050, incorporated herein by reference).

Standard recombinant DNA and molecular cloning techniques used herein are well known in the art and are described more fully in Sambrook et al. *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, 1989 (hereinafter “Maniatis”).

Nucleic acid fragments encoding at least a portion of several plant lecithin:cholesterol acyltransferases have been isolated and identified by comparison of random plant cDNA sequences to public databases containing nucleotide and protein sequences using the BLAST algorithms well known to those skilled in the art. The nucleic acid fragments of the instant invention may be used to isolate cDNAs and genes encoding homologous proteins from the same or other plant species. Isolation of homologous genes using sequence-dependent protocols is well known in the art. Examples of sequence-dependent protocols include, but are not limited to, methods of nucleic acid hybridization, and methods of DNA and RNA amplification as exemplified by various uses of nucleic acid amplification technologies (e.g., polymerase chain reaction, ligase chain reaction).

For example, genes encoding other plant lecithin:cholesterol acyltransferases, either as cDNAs or genomic DNAs, could be isolated directly by using all or a portion of the instant nucleic acid fragments as DNA hybridization probes to screen libraries from any desired plant employing methodology well known to those skilled in the art. Specific

5 oligonucleotide probes based upon the instant nucleic acid sequences can be designed and synthesized by methods known in the art (Maniatis). Moreover, the entire sequences can be used directly to synthesize DNA probes by methods known to the skilled artisan such as random primer DNA labeling, nick translation, or end-labeling techniques, or RNA probes using available *in vitro* transcription systems. In addition, specific primers can be designed
10 and used to amplify a part or all of the instant sequences. The resulting amplification products can be labeled directly during amplification reactions or labeled after amplification reactions, and used as probes to isolate full length cDNA or genomic fragments under conditions of appropriate stringency.

In addition, two short segments of the instant nucleic acid fragments may be used in
15 polymerase chain reaction protocols to amplify longer nucleic acid fragments encoding homologous genes from DNA or RNA. The polymerase chain reaction may also be performed on a library of cloned nucleic acid fragments wherein the sequence of one primer is derived from the instant nucleic acid fragments, and the sequence of the other primer takes advantage of the presence of the polyadenylic acid tracts to the 3' end of the mRNA
20 precursor encoding plant genes. Alternatively, the second primer sequence may be based upon sequences derived from the cloning vector. For example, the skilled artisan can follow the RACE protocol (Frohman et al. (1988) *Proc. Natl. Acad. Sci. USA* 85:8998-9002) to generate cDNAs by using PCR to amplify copies of the region between a single point in the transcript and the 3' or 5' end. Primers oriented in the 3' and 5' directions can be designed
25 from the instant sequences. Using commercially available 3' RACE or 5' RACE systems (BRL), specific 3' or 5' cDNA fragments can be isolated (Ohara et al. (1989) *Proc. Natl. Acad. Sci. USA* 86:5673-5677; Loh et al. (1989) *Science* 243:217-220). Products generated by the 3' and 5' RACE procedures can be combined to generate full-length cDNAs (Frohman and Martin (1989) *Techniques* 1:165). Consequently, a polynucleotide comprising a
30 nucleotide sequence of at least one of 60 (preferably one of at least 40, most preferably one of at least 30) contiguous nucleotides derived from a nucleotide sequence selected from the group consisting of SEQ ID NOs:1, 3, 5, 7, 9, 11, 13, and the complement of such nucleotide sequences may be used in such methods to obtain a nucleic acid fragment encoding a substantial portion of an amino acid sequence of a polypeptide. The present
35 invention relates to a method of obtaining a nucleic acid fragment encoding a substantial portion of a polypeptide of a gene (such as plant lecithin:cholesterol acyltransferases) preferably a substantial portion of a plant polypeptide of a gene, comprising the steps of: synthesizing an oligonucleotide primer comprising a nucleotide sequence of at least one of

60 (preferably at least one of 40, most preferably at least one of 30) contiguous nucleotides derived from a nucleotide sequence selected from the group consisting of SEQ ID NOs:1, 3, 5, 7, 9, 11, and 13, and the complement of such nucleotide sequences; and amplifying a nucleic acid fragment (preferably a cDNA inserted in a cloning vector) using the
5 oligonucleotide primer. The amplified nucleic acid fragment preferably will encode a portion of a polypeptide.

Availability of the instant nucleotide and deduced amino acid sequences facilitates immunological screening of cDNA expression libraries. Synthetic peptides representing portions of the instant amino acid sequences may be synthesized. These peptides can be
10 used to immunize animals to produce polyclonal or monoclonal antibodies with specificity for peptides or proteins comprising the amino acid sequences. These antibodies can be then be used to screen cDNA expression libraries to isolate full-length cDNA clones of interest (Lerner (1984) *Adv. Immunol.* 36:1-34; Maniatis).

The nucleic acid fragments of the instant invention may be used to create transgenic
15 plants in which the disclosed polypeptides are present at higher or lower levels than normal or in cell types or developmental stages in which they are not normally found. This would have the effect of altering the level of, and composition of, plant sterols in those cells. Since plant sterols help regulate membrane fluidity, this may be beneficial in regulating cold or heat tolerance in plants. Also, it is believed that lecithin is the acyl donor for this reaction,
20 therefore the levels of lecithin found in the plants could be altered by varying the activity of LCAT.

Overexpression of the proteins of the instant invention may be accomplished by first constructing a chimeric gene in which the coding region is operably linked to a promoter capable of directing expression of a gene in the desired tissues at the desired stage of
25 development. For reasons of convenience, the chimeric gene may comprise promoter sequences and translation leader sequences derived from the same genes. 3' Non-coding sequences encoding transcription termination signals may also be provided. The instant chimeric gene may also comprise one or more introns in order to facilitate gene expression.

Plasmid vectors comprising the instant chimeric gene can then be constructed. The
30 choice of plasmid vector is dependent upon the method that will be used to transform host plants. The skilled artisan is well aware of the genetic elements that must be present on the plasmid vector in order to successfully transform, select and propagate host cells containing the chimeric gene. The skilled artisan will also recognize that different independent transformation events will result in different levels and patterns of expression (Jones et al.
35 (1985) *EMBO J.* 4:2411-2418; De Almeida et al. (1989) *Mol. Gen. Genetics* 218:78-86), and thus that multiple events must be screened in order to obtain lines displaying the desired expression level and pattern. Such screening may be accomplished by Southern analysis of

DNA, Northern analysis of mRNA expression, Western analysis of protein expression, or phenotypic analysis.

For some applications it may be useful to direct the instant polypeptide to different cellular compartments, or to facilitate its secretion from the cell. It is thus envisioned that the chimeric gene described above may be further supplemented by altering the coding sequence to encode the instant polypeptide with appropriate intracellular targeting sequences such as transit sequences (Keegstra (1989) *Cell* 56:247-253), signal sequences or sequences encoding endoplasmic reticulum localization (Chrispeels (1991) *Ann. Rev. Plant Phys. Plant Mol. Biol.* 42:21-53), or nuclear localization signals (Raikhel (1992) *Plant Phys.*

100:1627-1632) added and/or with targeting sequences that are already present removed. While the references cited give examples of each of these, the list is not exhaustive and more targeting signals of utility may be discovered in the future.

It may also be desirable to reduce or eliminate expression of genes encoding the instant polypeptides in plants for some applications. In order to accomplish this, a chimeric gene designed for co-suppression of the instant polypeptide can be constructed by linking a gene or gene fragment encoding that polypeptide to plant promoter sequences.

Alternatively, a chimeric gene designed to express antisense RNA for all or part of the instant nucleic acid fragment can be constructed by linking the gene or gene fragment in reverse orientation to plant promoter sequences. Either the co-suppression or antisense chimeric genes could be introduced into plants via transformation wherein expression of the corresponding endogenous genes are reduced or eliminated.

Molecular genetic solutions to the generation of plants with altered gene expression have a decided advantage over more traditional plant breeding approaches. Changes in plant phenotypes can be produced by specifically inhibiting expression of one or more genes by antisense inhibition or cosuppression (U.S. Patent Nos. 5,190,931, 5,107,065 and 5,283,323). An antisense or cosuppression construct would act as a dominant negative regulator of gene activity. While conventional mutations can yield negative regulation of gene activity these effects are most likely recessive. The dominant negative regulation available with a transgenic approach may be advantageous from a breeding perspective. In addition, the ability to restrict the expression of specific phenotype to the reproductive tissues of the plant by the use of tissue specific promoters may confer agronomic advantages relative to conventional mutations which may have an effect in all tissues in which a mutant gene is ordinarily expressed.

The person skilled in the art will know that special considerations are associated with the use of antisense or cosuppression technologies in order to reduce expression of particular genes. For example, the proper level of expression of sense or antisense genes may require the use of different chimeric genes utilizing different regulatory elements known to the skilled artisan. Once transgenic plants are obtained by one of the methods described above,

it will be necessary to screen individual transgenics for those that most effectively display the desired phenotype. Accordingly, the skilled artisan will develop methods for screening large numbers of transformants. The nature of these screens will generally be chosen on practical grounds, and is not an inherent part of the invention. For example, one can screen by looking for changes in gene expression by using antibodies specific for the protein encoded by the gene being suppressed, or one could establish assays that specifically measure enzyme activity. A preferred method will be one which allows large numbers of samples to be processed rapidly, since it will be expected that a large number of transformants will be negative for the desired phenotype.

The instant polypeptide (or portions thereof) may be produced in heterologous host cells, particularly in the cells of microbial hosts, and can be used to prepare antibodies to these proteins by methods well known to those skilled in the art. The antibodies are useful for detecting the polypeptide of the instant invention *in situ* in cells or *in vitro* in cell extracts. Preferred heterologous host cells for production of the instant polypeptide are microbial hosts. Microbial expression systems and expression vectors containing regulatory sequences that direct high level expression of foreign proteins are well known to those skilled in the art. Any of these could be used to construct a chimeric gene for production of the instant polypeptide. This chimeric gene could then be introduced into appropriate microorganisms via transformation to provide high level expression of the encoded plant lecithin:cholesterol acyltransferases. An example of a vector for high level expression of the instant polypeptide in a bacterial host is provided (Example 6).

Additionally, the instant polypeptide can be used as a targets to facilitate design and/or identification of inhibitors of those enzymes that may be useful as herbicides. This is desirable because the polypeptide described herein catalyze steps in sterol modification in plants. The composition of plant sterols are important factors in a plants ability to adapt to temperature changes in the environment, shifts in sunlight, and drought stress. In addition, the sterol derivative hormones, such as brassinosteroids, can affect overall growth and development of plants (Szekeres et al. (1996) *Cell* 85:171-182; Clouse and Sasse (1998) *Ann Rev Plant Physiol Plant Mol Biol* 49:427-451). Brassinosteroid production may be affected by changes in composition of membrane sterols. Accordingly, inhibition of the activity of one or more of the enzymes described herein could lead to inhibition of plant growth. Thus, the instant polypeptide could be appropriate for new herbicide discovery and design.

All or a substantial portion of the nucleic acid fragments of the instant invention may also be used as probes for genetically and physically mapping the genes that they are a part of, and as markers for traits linked to those genes. Such information may be useful in plant breeding in order to develop lines with desired phenotypes. For example, the instant nucleic acid fragments may be used as restriction fragment length polymorphism (RFLP) markers. Southern blots (Maniatis) of restriction-digested plant genomic DNA may be probed with

the nucleic acid fragments of the instant invention. The resulting banding patterns may then be subjected to genetic analyses using computer programs such as MapMaker (Lander et al. (1987) *Genomics* 1:174-181) in order to construct a genetic map. In addition, the nucleic acid fragments of the instant invention may be used to probe Southern blots containing restriction endonuclease-treated genomic DNAs of a set of individuals representing parent and progeny of a defined genetic cross. Segregation of the DNA polymorphisms is noted and used to calculate the position of the instant nucleic acid sequence in the genetic map previously obtained using this population (Botstein et al. (1980) *Am. J. Hum. Genet.* 32:314-331).

The production and use of plant gene-derived probes for use in genetic mapping is described in Bernatzky and Tanksley (1986) *Plant Mol. Biol. Reporter* 4:37-41. Numerous publications describe genetic mapping of specific cDNA clones using the methodology outlined above or variations thereof. For example, F2 intercross populations, backcross populations, randomly mated populations, near isogenic lines, and other sets of individuals may be used for mapping. Such methodologies are well known to those skilled in the art.

Nucleic acid probes derived from the instant nucleic acid sequences may also be used for physical mapping (i.e., placement of sequences on physical maps; see Hoheisel et al. In: *Nonmammalian Genomic Analysis: A Practical Guide*, Academic press 1996, pp. 319-346, and references cited therein).

In another embodiment, nucleic acid probes derived from the instant nucleic acid sequences may be used in direct fluorescence *in situ* hybridization (FISH) mapping (Trask (1991) *Trends Genet.* 7:149-154). Although current methods of FISH mapping favor use of large clones (several to several hundred KB; see Laan et al. (1995) *Genome Res.* 5:13-20), improvements in sensitivity may allow performance of FISH mapping using shorter probes.

A variety of nucleic acid amplification-based methods of genetic and physical mapping may be carried out using the instant nucleic acid sequences. Examples include allele-specific amplification (Kazazian (1989) *J. Lab. Clin. Med.* 11:95-96), polymorphism of PCR-amplified fragments (CAPS; Sheffield et al. (1993) *Genomics* 16:325-332), allele-specific ligation (Landegren et al. (1988) *Science* 241:1077-1080), nucleotide extension reactions (Sokolov (1990) *Nucleic Acid Res.* 18:3671), Radiation Hybrid Mapping (Walter et al. (1997) *Nat. Genet.* 7:22-28) and Happy Mapping (Dear and Cook (1989) *Nucleic Acid Res.* 17:6795-6807). For these methods, the sequence of a nucleic acid fragment is used to design and produce primer pairs for use in the amplification reaction or in primer extension reactions. The design of such primers is well known to those skilled in the art. In methods employing PCR-based genetic mapping, it may be necessary to identify DNA sequence differences between the parents of the mapping cross in the region corresponding to the instant nucleic acid sequence. This, however, is generally not necessary for mapping methods.

Loss of function mutant phenotypes may be identified for the instant cDNA clones either by targeted gene disruption protocols or by identifying specific mutants for these genes contained in a maize population carrying mutations in all possible genes (Ballinger and Benzer (1989) *Proc. Natl. Acad. Sci USA* 86:9402-9406; Koes et al. (1995) *Proc. Natl. Acad. Sci USA* 92:8149-8153; Bensen et al. (1995) *Plant Cell* 7:75-84). The latter approach may be accomplished in two ways. First, short segments of the instant nucleic acid fragments may be used in polymerase chain reaction protocols in conjunction with a mutation tag sequence primer on DNAs prepared from a population of plants in which Mutator transposons or some other mutation-causing DNA element has been introduced (see Bensen, *supra*). The amplification of a specific DNA fragment with these primers indicates the insertion of the mutation tag element in or near the plant gene encoding the instant polypeptide. Alternatively, the instant nucleic acid fragment may be used as a hybridization probe against PCR amplification products generated from the mutation population using the mutation tag sequence primer in conjunction with an arbitrary genomic site primer, such as that for a restriction enzyme site-anchored synthetic adaptor. With either method, a plant containing a mutation in the endogenous gene encoding the instant polypeptide can be identified and obtained. This mutant plant can then be used to determine or confirm the natural function of the instant polypeptide disclosed herein.

EXAMPLES

The present invention is further defined in the following Examples, in which all parts and percentages are by weight and degrees are Celsius, unless otherwise stated. It should be understood that these Examples, while indicating preferred embodiments of the invention, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions.

EXAMPLE 1

Composition of cDNA Libraries: Isolation and Sequencing of cDNA Clones

cDNA libraries representing mRNAs from various corn and soybean tissues were prepared. The characteristics of the libraries are described below.

TABLE 2
cDNA Libraries from Corn and Soybean

Library	Tissue	Clone
cbn10	Corn Developing Kernel 10 Days After Pollination	cbn10.pk0052.g10
cbn2	Corn Developing Kernel Two Days After Pollination	cbn2.pk0017.h4
p0016	Corn Embryo 13 Days After Pollination	p0016.ctsbo30r
p0018	Corn Ear Shoot	p0018.chstj36r
cen3n	Corn Endosperm 20 Days After Pollination*	cen3n.pk0141.f2:fis
ceplc	Corn (<i>Zea mays</i> L.) pollinated (25 hrs after pollination, 48-72 after emergence) ears	ceplc.pk001.f7:fis
chpc24	Corn (MBS847) 8 Day Old Shoot Treated 24 Hours With PDO Herbicide MK593**	chpc24.pk0001.c1
sl2	Soybean Two-Week-Old Developing Seedlings Treated With 2.5 ppm chlorimuron	sl2.pk0015.e8:fis

*This library was normalized essentially as described in U.S. Patent No. 5,482,845, incorporated herein by reference.

5 **Application of 2-[(2,4-dihydro-2,6,9-trimethyl[1]benzothiopyrano[4,3-c]pyrazol-8-yl)carbonyl]-1,3-cyclohexanedione *S,S*-dioxide; synthesis and methods of using this compound are described in WO 97/19087, incorporated herein by reference.

cDNA libraries may be prepared by any one of many methods available. For example, the cDNAs may be introduced into plasmid vectors by first preparing the cDNA libraries in Uni-ZAP™ XR vectors according to the manufacturer's protocol (Stratagene Cloning Systems, La Jolla, CA). The Uni-ZAP™ XR libraries are converted into plasmid libraries according to the protocol provided by Stratagene. Upon conversion, cDNA inserts will be contained in the plasmid vector pBluescript. In addition, the cDNAs may be introduced directly into precut Bluescript II SK(+) vectors (Stratagene) using T4 DNA ligase (New England Biolabs), followed by transfection into DH10B cells according to the manufacturer's protocol (GIBCO BRL Products). Once the cDNA inserts are in plasmid vectors, plasmid DNAs are prepared from randomly picked bacterial colonies containing recombinant pBluescript plasmids, or the insert cDNA sequences are amplified via polymerase chain reaction using primers specific for vector sequences flanking the inserted cDNA sequences. Amplified insert DNAs or plasmid DNAs are sequenced in dye-primer sequencing reactions to generate partial cDNA sequences (expressed sequence tags or "ESTs"; see Adams et al., (1991) *Science* 252:1651-1656). The resulting ESTs are analyzed using a Perkin Elmer Model 377 fluorescent sequencer.

EXAMPLE 2

Identification of cDNA Clones

cDNA clones encoding plant lecithin:cholesterol acyltransferases were identified by conducting BLAST (Basic Local Alignment Search Tool; Altschul et al. (1993) *J. Mol. Biol.*

2/5:403-410; see also www.ncbi.nlm.nih.gov/BLAST/) searches for similarity to sequences contained in the BLAST "nr" database (comprising all non-redundant GenBank CDS translations, sequences derived from the 3-dimensional structure Brookhaven Protein Data Bank, the last major release of the SWISS-PROT protein sequence database, EMBL, and DDBJ databases). The cDNA sequences obtained in Example 1 were analyzed for similarity to all publicly available DNA sequences contained in the "nr" database using the BLASTN algorithm provided by the National Center for Biotechnology Information (NCBI). The DNA sequences were translated in all reading frames and compared for similarity to all publicly available protein sequences contained in the "nr" database using the BLASTX algorithm (Gish and States (1993) *Nat. Genet.* 3:266-272) provided by the NCBI. For convenience, the P-value (probability) of observing a match of a cDNA sequence to a sequence contained in the searched databases merely by chance as calculated by BLAST are reported herein as "pLog" values, which represent the negative of the logarithm of the reported P-value. Accordingly, the greater the pLog value, the greater the likelihood that the cDNA sequence and the BLAST "hit" represent homologous proteins.

EXAMPLE 3

Characterization of cDNA Clones Encoding Lecithin:Cholesterol Acyltransferases

The BLASTX search using the nucleotide sequences from clones cep1c.pk001.f7 and sl2.pk0015.e8 and the nucleotide sequences from the contig assembled of clones chpc24.pk0001.c1, cbn2.pk0017.h4, cbn10.pk0052.g10, p0016.ctsbo30r and p0018.chstj36r revealed similarity of the proteins encoded by the cDNAs to lecithin:cholesterol acyltransferase from *Homo sapiens* (NCBI gi Accession No. 998999) and from *rattus norvegicus* (NCBI gi Accession No. 418623). The BLAST results for each of these sequences are shown in Table 3:

TABLE 3

BLAST Results for Clones Encoding Polypeptides Homologous
to Lecithin:Cholesterol Acyltransferases

Clone	Status	Blast pLog Score	
		98999	418623
cep1c.pk001.f7	EST	16.40	13.40
Contig of:	Contig	12.10	14.00
chpc24.pk0001.c1			
cbn2.pk0017.h4			
cbn10.pk0052.g10			
p0016.ctsbo30r			
p0018.chstj36r			
sl2.pk0015.e8	EST	44.00	42.05

The sequence of a portion of the cDNA insert from clone cep1c.pk001.f7 is shown in SEQ ID NO:1; the deduced amino acid sequence of this cDNA is shown in SEQ ID NO:2.

The sequence of a contig assembled of the cDNA inserts from clones chpc24.pk0001.c1, cbn2.pk0017.h4, cbn10.pk0052.g10, p0016.ctsbo30r and p0018.chstj36r is shown in SEQ ID NO:3; the deduced amino acid sequence of this contig is shown in SEQ ID NO:4. The sequence of the entire cDNA insert in clone sl2.pk0015.e8 was determined and is shown in SEQ ID NO:5; the deduced amino acid sequence of this cDNA is shown in SEQ ID NO:6. The amino acid sequence set forth in SEQ ID NO:6 was evaluated by BLASTP, yielding a pLog value of 47.30 versus the *Homo sapiens* sequence. The soybean lecithin:cholesterol acyltransferase is 32.2% identical to the *Homo sapiens* sequence. Sequence percent identity calculations were performed by the Jotun Hein method (Hein, J. J. (1990) *Meth. Enz.* 183:626-645) using the Megalign program of the LASARGENE bioinformatics computing suite (DNASTAR Inc., Madison, WI) with the default parameters for pairwise alignment (ktuple=2). BLAST scores and probabilities indicate that the instant nucleic acid fragments encode portions of corn lecithin:cholesterol acyltransferase and an almost entire soybean lecithin:cholesterol acyltransferases. These sequences represent the first plant sequences encoding lecithin:cholesterol acyltransferases.

After the filing of the provisional application (U.S. Provisional Application No. 60/110782, filed December 3, 1998) an *Arabidopsis thaliana* gene was discovered in the NCBI database (with a deposit date of December 1, 1998). The BLASTX search using the EST sequences from clones listed in Table 4 revealed similarity of the polypeptides encoded by the cDNAs to lecithin:cholesterol acyltransferases from *Arabidopsis thaliana* (NCBI Accession No. gi 3935185). Shown in Table 4 are the BLAST results for individual ESTs ("EST"), the sequences of the entire cDNA inserts comprising the indicated cDNA clones ("FIS"), contigs assembled from two or more ESTs ("Contig"), contigs assembled from an FIS and one or more ESTs ("Contig*"), or sequences encoding the entire protein derived from an FIS, a contig, or an FIS and PCR ("CGS"):

TABLE 4
BLAST Results for Sequences Encoding Polypeptides Homologous
to Lecithin:Cholesterol Acyltransferases

Clone	Status	BLAST pLog Score 3935185
cen3n.pk0141.f2: fis	CGS	63.70
ceplc.pk001.f7: fis	CGS	84.00
chpc24.pk0001.c1	FIS	64.52
sl2.pk0015.e8: fis	CGS	152.00

Figure 1 presents an alignment of the amino acid sequences set forth in SEQ ID NOs:8, 10, 12, and 14, and the *Arabidopsis thaliana* sequence (SEQ ID NO:15). The data in

Table 4 represents a calculation of the percent identity of the amino acid sequences set forth in SEQ ID NOs:8, 10, 12, and 14, and the *Arabidopsis thaliana* sequence (SEQ ID NO:15).

TABLE 4

5 Percent Identity of Amino Acid Sequences Deduced From the Nucleotide Sequences of cDNA Clones Encoding Polypeptides Homologous to Lecithin:Cholesterol Acyltransferases

SEQ ID NO.	Percent Identity to 3935185
8	29.4%
10	37.2%
12	28.5%
14	57.6%

Sequence alignments and percent identity calculations were performed using the Megalign program of the LASERGENE bioinformatics computing suite (DNASTAR Inc., Madison, WI). Multiple alignment of the sequences was performed using the Clustal method of alignment (Higgins and Sharp (1989) *CABIOS*, 5:151-153) with the default parameters (GAP PENALTY=10, GAP LENGTH PENALTY=10). Default parameters for pairwise alignments using the Clustal method were KTUPLE 1, GAP PENALTY=3, WINDOW=5 and DIAGONALS SAVED=5. Sequence alignments and BLAST scores and probabilities indicate that the nucleic acid fragments comprising the instant cDNA clones encode a substantial portion of a lecithin:cholesterol acyltransferases. These sequences represent the first plant sequences encoding lecithin:cholesterol acyltransferases.

EXAMPLE 4

20 Expression of Chimeric Genes in Monocot Cells

A chimeric gene comprising a cDNA encoding the instant polypeptide in sense orientation with respect to the maize 27 kD zein promoter that is located 5' to the cDNA fragment, and the 10 kD zein 3' end that is located 3' to the cDNA fragment, can be constructed. The cDNA fragment of this gene may be generated by polymerase chain reaction (PCR) of the cDNA clone using appropriate oligonucleotide primers. Cloning sites (NcoI or SmaI) can be incorporated into the oligonucleotides to provide proper orientation of the DNA fragment when inserted into the digested vector pML103 as described below. Amplification is then performed in a standard PCR. The amplified DNA is then digested with restriction enzymes NcoI and SmaI and fractionated on an agarose gel. The appropriate band can be isolated from the gel and combined with a 4.9 kb NcoI-SmaI fragment of the plasmid pML103. Plasmid pML103 has been deposited under the terms of the Budapest Treaty at ATCC (American Type Culture Collection, 10801 University Blvd., Manassas, VA 20110-2209), and bears accession number ATCC 97366. The DNA segment from

pML103 contains a 1.05 kb Sall-NcoI promoter fragment of the maize 27 kD zein gene and a 0.96 kb SmaI-Sall fragment from the 3' end of the maize 10 kD zein gene in the vector pGem9Zf(+) (Promega). Vector and insert DNA can be ligated at 15°C overnight, essentially as described (Maniatis). The ligated DNA may then be used to transform *E. coli* XL1-Blue (Epicurian Coli XL-1 Blue™; Stratagene). Bacterial transformants can be screened by restriction enzyme digestion of plasmid DNA and limited nucleotide sequence analysis using the dideoxy chain termination method (Sequenase™ DNA Sequencing Kit; U.S. Biochemical). The resulting plasmid construct would comprise a chimeric gene encoding, in the 5' to 3' direction, the maize 27 kD zein promoter, a cDNA fragment encoding the instant polypeptide, and the 10 kD zein 3' region.

The chimeric gene described above can then be introduced into corn cells by the following procedure. Immature corn embryos can be dissected from developing caryopses derived from crosses of the inbred corn lines H99 and LH132. The embryos are isolated 10 to 11 days after pollination when they are 1.0 to 1.5 mm long. The embryos are then placed with the axis-side facing down and in contact with agarose-solidified N6 medium (Chu et al. (1975) *Sci. Sin. Peking* 18:659-668). The embryos are kept in the dark at 27°C. Friable embryogenic callus consisting of undifferentiated masses of cells with somatic proembryoids and embryoids borne on suspensor structures proliferates from the scutellum of these immature embryos. The embryogenic callus isolated from the primary explant can be cultured on N6 medium and sub-cultured on this medium every 2 to 3 weeks.

The plasmid, p35S/Ac (obtained from Dr. Peter Eckes, Hoechst Ag, Frankfurt, Germany) may be used in transformation experiments in order to provide for a selectable marker. This plasmid contains the *Pat* gene (see European Patent Publication 0 242 236) which encodes phosphinothricin acetyl transferase (PAT). The enzyme PAT confers resistance to herbicidal glutamine synthetase inhibitors such as phosphinothricin. The *pat* gene in p35S/Ac is under the control of the 35S promoter from Cauliflower Mosaic Virus (Odell et al. (1985) *Nature* 313:810-812) and the 3' region of the nopaline synthase gene from the T-DNA of the Ti plasmid of *Agrobacterium tumefaciens*.

The particle bombardment method (Klein et al. (1987) *Nature* 327:70-73) may be used to transfer genes to the callus culture cells. According to this method, gold particles (1 µm in diameter) are coated with DNA using the following technique. Ten µg of plasmid DNAs are added to 50 µL of a suspension of gold particles (60 mg per mL). Calcium chloride (50 µL of a 2.5 M solution) and spermidine free base (20 µL of a 1.0 M solution) are added to the particles. The suspension is vortexed during the addition of these solutions. After 10 minutes, the tubes are briefly centrifuged (5 sec at 15,000 rpm) and the supernatant removed. The particles are resuspended in 200 µL of absolute ethanol, centrifuged again and the supernatant removed. The ethanol rinse is performed again and the particles resuspended in a final volume of 30 µL of ethanol. An aliquot (5 µL) of the DNA-coated

gold particles can be placed in the center of a Kapton™ flying disc (Bio-Rad Labs). The particles are then accelerated into the corn tissue with a Biolistic™ PDS-1000/He (Bio-Rad Instruments, Hercules CA), using a helium pressure of 1000 psi, a gap distance of 0.5 cm and a flying distance of 1.0 cm.

5 For bombardment, the embryogenic tissue is placed on filter paper over agarose-solidified N6 medium. The tissue is arranged as a thin lawn and covered a circular area of about 5 cm in diameter. The petri dish containing the tissue can be placed in the chamber of the PDS-1000/He approximately 8 cm from the stopping screen. The air in the chamber is then evacuated to a vacuum of 28 inches of Hg. The macrocarrier is accelerated with a
10 helium shock wave using a rupture membrane that bursts when the He pressure in the shock tube reaches 1000 psi.

Seven days after bombardment the tissue can be transferred to N6 medium that contains glufosinate (2 mg per liter) and lacks casein or proline. The tissue continues to grow slowly on this medium. After an additional 2 weeks the tissue can be transferred to
15 fresh N6 medium containing glufosinate. After 6 weeks, areas of about 1 cm in diameter of actively growing callus can be identified on some of the plates containing the glufosinate-supplemented medium. These calli may continue to grow when sub-cultured on the selective medium.

Plants can be regenerated from the transgenic callus by first transferring clusters of
20 tissue to N6 medium supplemented with 0.2 mg per liter of 2,4-D. After two weeks the tissue can be transferred to regeneration medium (Fromm et al. (1990) *Bio/Technology* 8:833-839).

EXAMPLE 5

Expression of Chimeric Genes in Dicot Cells

25 A seed-specific expression cassette composed of the promoter and transcription terminator from the gene encoding the β subunit of the seed storage protein phaseolin from the bean *Phaseolus vulgaris* (Doyle et al. (1986) *J. Biol. Chem.* 261:9228-9238) can be used for expression of the instant polypeptide in transformed soybean. The phaseolin cassette includes about 500 nucleotides upstream (5') from the translation initiation codon and about
30 1650 nucleotides downstream (3') from the translation stop codon of phaseolin. Between the 5' and 3' regions are the unique restriction endonuclease sites Nco I (which includes the ATG translation initiation codon), Sma I, Kpn I and Xba I. The entire cassette is flanked by Hind III sites.

The cDNA fragment of this gene may be generated by polymerase chain reaction
35 (PCR) of the cDNA clone using appropriate oligonucleotide primers. Cloning sites can be incorporated into the oligonucleotides to provide proper orientation of the DNA fragment when inserted into the expression vector. Amplification is then performed as described

above, and the isolated fragment is inserted into a pUC18 vector carrying the seed expression cassette.

Soybean embryos may then be transformed with the expression vector comprising sequences encoding the instant polypeptide. To induce somatic embryos, cotyledons, 3-5 mm in length dissected from surface sterilized, immature seeds of the soybean cultivar A2872, can be cultured in the light or dark at 26°C on an appropriate agar medium for 6-10 weeks. Somatic embryos which produce secondary embryos are then excised and placed into a suitable liquid medium. After repeated selection for clusters of somatic embryos which multiplied as early, globular staged embryos, the suspensions are maintained as described below.

Soybean embryogenic suspension cultures can be maintained in 35 mL liquid media on a rotary shaker, 150 rpm, at 26°C with fluorescent lights on a 16:8 hour day/night schedule. Cultures are subcultured every two weeks by inoculating approximately 35 mg of tissue into 35 mL of liquid medium.

Soybean embryogenic suspension cultures may then be transformed by the method of particle gun bombardment (Klein et al. (1987) *Nature* (London) 327:70-73, U.S. Patent No. 4,945,050). A DuPont Biolistic™ PDS1000/HE instrument (helium retrofit) can be used for these transformations.

A selectable marker gene which can be used to facilitate soybean transformation is a chimeric gene composed of the 35S promoter from Cauliflower Mosaic Virus (Odell et al. (1985) *Nature* 313:810-812), the hygromycin phosphotransferase gene from plasmid pJR225 (from *E. coli*; Gritz et al. (1983) *Gene* 25:179-188) and the 3' region of the nopaline synthase gene from the T-DNA of the Ti plasmid of *Agrobacterium tumefaciens*. The seed expression cassette comprising the phaseolin 5' region, the fragment encoding the instant polypeptide and the phaseolin 3' region can be isolated as a restriction fragment. This fragment can then be inserted into a unique restriction site of the vector carrying the marker gene.

To 50 µL of a 60 mg/mL 1 µm gold particle suspension is added (in order): 5 µL DNA (1 µg/µL), 20 µL spermidine (0.1 M), and 50 µL CaCl₂ (2.5 M). The particle preparation is then agitated for three minutes, spun in a microfuge for 10 seconds and the supernatant removed. The DNA-coated particles are then washed once in 400 µL 70% ethanol and resuspended in 40 µL of anhydrous ethanol. The DNA/particle suspension can be sonicated three times for one second each. Five µL of the DNA-coated gold particles are then loaded on each macro carrier disk.

Approximately 300-400 mg of a two-week-old suspension culture is placed in an empty 60x15 mm petri dish and the residual liquid removed from the tissue with a pipette. For each transformation experiment, approximately 5-10 plates of tissue are normally bombarded. Membrane rupture pressure is set at 1100 psi and the chamber is evacuated to a vacuum of 28 inches mercury. The tissue is placed approximately 3.5 inches away from the

retaining screen and bombarded three times. Following bombardment, the tissue can be divided in half and placed back into liquid and cultured as described above.

Five to seven days post bombardment, the liquid media may be exchanged with fresh media, and eleven to twelve days post bombardment with fresh media containing 50 mg/mL hygromycin. This selective media can be refreshed weekly. Seven to eight weeks post bombardment, green, transformed tissue may be observed growing from untransformed, necrotic embryogenic clusters. Isolated green tissue is removed and inoculated into individual flasks to generate new, clonally propagated, transformed embryogenic suspension cultures. Each new line may be treated as an independent transformation event. These suspensions can then be subcultured and maintained as clusters of immature embryos or regenerated into whole plants by maturation and germination of individual somatic embryos.

EXAMPLE 6

Expression of Chimeric Genes in Microbial Cells

The cDNAs encoding the instant polypeptide can be inserted into the T7 *E. coli* expression vector pBT430. This vector is a derivative of pET-3a (Rosenberg et al. (1987) *Gene* 56:125-135) which employs the bacteriophage T7 RNA polymerase/T7 promoter system. Plasmid pBT430 was constructed by first destroying the EcoR I and Hind III sites in pET-3a at their original positions. An oligonucleotide adaptor containing EcoR I and Hind III sites was inserted at the BamH I site of pET-3a. This created pET-3aM with additional unique cloning sites for insertion of genes into the expression vector. Then, the Nde I site at the position of translation initiation was converted to an Nco I site using oligonucleotide-directed mutagenesis. The DNA sequence of pET-3aM in this region, 5'-CATATGG, was converted to 5'-CCCATGG in pBT430.

Plasmid DNA containing a cDNA may be appropriately digested to release a nucleic acid fragment encoding the protein. This fragment may then be purified on a 1% NuSieve GTG™ low melting agarose gel (FMC). Buffer and agarose contain 10 µg/ml ethidium bromide for visualization of the DNA fragment. The fragment can then be purified from the agarose gel by digestion with GELase™ (Epicentre Technologies) according to the manufacturer's instructions, ethanol precipitated, dried and resuspended in 20 µL of water. Appropriate oligonucleotide adapters may be ligated to the fragment using T4 DNA ligase (New England Biolabs, Beverly, MA). The fragment containing the ligated adapters can be purified from the excess adapters using low melting agarose as described above. The vector pBT430 is digested, dephosphorylated with alkaline phosphatase (NEB) and deproteinized with phenol/chloroform as described above. The prepared vector pBT430 and fragment can then be ligated at 16°C for 15 hours followed by transformation into DH5 electrocompetent cells (GIBCO BRL). Transformants can be selected on agar plates containing LB media and 100 µg/mL ampicillin. Transformants containing the gene encoding the instant polypeptide

are then screened for the correct orientation with respect to the T7 promoter by restriction enzyme analysis.

For high level expression, a plasmid clone with the cDNA insert in the correct orientation relative to the T7 promoter can be transformed into *E. coli* strain BL21(DE3) (Studier et al. (1986) *J. Mol. Biol.* 189:113-130). Cultures are grown in LB medium containing ampicillin (100 mg/L) at 25°C. At an optical density at 600 nm of approximately 1, IPTG (isopropylthio- β -galactoside, the inducer) can be added to a final concentration of 0.4 mM and incubation can be continued for 3 h at 25°. Cells are then harvested by centrifugation and re-suspended in 50 μ L of 50 mM Tris-HCl at pH 8.0 containing 0.1 mM DTT and 0.2 mM phenyl methylsulfonyl fluoride. A small amount of 1 mm glass beads can be added and the mixture sonicated 3 times for about 5 seconds each time with a microprobe sonicator. The mixture is centrifuged and the protein concentration of the supernatant determined. One μ g of protein from the soluble fraction of the culture can be separated by SDS-polyacrylamide gel electrophoresis. Gels can be observed for protein bands migrating at the expected molecular weight.

EXAMPLE 7

Evaluating Compounds for Their Ability to Inhibit the Activity of Lecithin:Cholesterol Acyltransferases

The polypeptide described herein may be produced using any number of methods known to those skilled in the art. Such methods include, but are not limited to, expression in bacteria as described in Example 6, or expression in eukaryotic cell culture, *in planta*, and using viral expression systems in suitably infected organisms or cell lines. The instant polypeptide may be expressed either as mature forms of the proteins as observed *in vivo* or as fusion proteins by covalent attachment to a variety of enzymes, proteins or affinity tags. Common fusion protein partners include glutathione S-transferase ("GST"), thioredoxin ("Trx"), maltose binding protein, and C- and/or N-terminal hexahistidine polypeptide ("His₆"). The fusion proteins may be engineered with a protease recognition site at the fusion point so that fusion partners can be separated by protease digestion to yield intact mature enzyme. Examples of such proteases include thrombin, enterokinase and factor Xa. However, any protease can be used which specifically cleaves the peptide connecting the fusion protein and the enzyme.

Purification of the instant polypeptide, if desired, may utilize any number of separation technologies familiar to those skilled in the art of protein purification. Examples of such methods include, but are not limited to, homogenization, filtration, centrifugation, heat denaturation, ammonium sulfate precipitation, desalting, pH precipitation, ion exchange chromatography, hydrophobic interaction chromatography and affinity chromatography, wherein the affinity ligand represents a substrate, substrate analog or inhibitor. When the instant polypeptide are expressed as fusion proteins, the purification protocol may include

the use of an affinity resin which is specific for the fusion protein tag attached to the expressed enzyme or an affinity resin containing ligands which are specific for the enzyme. For example, the instant polypeptide may be expressed as a fusion protein coupled to the C-terminus of thioredoxin. In addition, a (His)₆ peptide may be engineered into the N-terminus of the fused thioredoxin moiety to afford additional opportunities for affinity purification. Other suitable affinity resins could be synthesized by linking the appropriate ligands to any suitable resin such as Sepharose-4B. In an alternate embodiment, a thioredoxin fusion protein may be eluted using dithiothreitol; however, elution may be accomplished using other reagents which interact to displace the thioredoxin from the resin. These reagents include β -mercaptoethanol or other reduced thiol. The eluted fusion protein may be subjected to further purification by traditional means as stated above, if desired. Proteolytic cleavage of the thioredoxin fusion protein and the enzyme may be accomplished after the fusion protein is purified or while the protein is still bound to the ThioBond™ affinity resin or other resin.

Crude, partially purified or purified enzyme, either alone or as a fusion protein, may be utilized in assays for the evaluation of compounds for their ability to inhibit enzymatic activation of the instant polypeptide disclosed herein. Assays may be conducted under well known experimental conditions which permit optimal enzymatic activity. For example, assays for lecithin:cholesterol acyltransferases are presented by Manabe, M. et al. (1987) *J. Lipid Res.* 28:1206-1215.

Various modifications of the invention in addition to those shown and described herein will be apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

The disclosure of each reference set forth above is incorporated herein by reference in its entirety.

CLAIMS

What is claimed is:

1. An isolated polynucleotide comprising a first nucleotide sequence encoding a polypeptide of at least 50 amino acids that has at least 60% identity based on the Clustal method of alignment when compared to a polypeptide selected from the group consisting of a plant lecithin:cholesterol acyltransferases polypeptide of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, and SEQ ID NO:14, or a second nucleotide sequence comprising the complement of the first nucleotide sequence.
2. The isolated polynucleotide of Claim 1, wherein the first nucleotide sequence consists of a nucleic acid sequence selected from the group consisting of SEQ ID NOs:1, 3, 5, 7, 9, 11, and 13 that codes for the polypeptide selected from the group consisting of SEQ ID NOs:2, 4, 6, 8, 10, 12, and 14.
3. The isolated polynucleotide of Claim 1 wherein the nucleotide sequences are DNA.
4. The isolated polynucleotide of Claim 1 wherein the nucleotide sequences are RNA.
5. A chimeric gene comprising the isolated polynucleotide of Claim 1 operably linked to suitable regulatory sequences.
6. An isolated host cell comprising the chimeric gene of Claim 5.
7. An isolated host cell comprising an isolated polynucleotide of Claim 1 or Claim 3.
8. The isolated host cell of Claim 7 wherein the isolated host is selected from the group consisting of yeast, bacteria, plant, and virus.
9. A virus comprising the isolated polynucleotide of Claim 1.
10. A polypeptide of at least 50 amino acids that has at least 60% identity based on the Clustal method of alignment when compared to a polypeptide selected from the group consisting of a plant lecithin:cholesterol acyltransferases polypeptide of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, and SEQ ID NO:14.
11. A method of selecting an isolated polynucleotide that affects the level of expression of a plant lecithin:cholesterol acyltransferases polypeptide in a plant cell, the method comprising the steps of:
 - (a) constructing an isolated polynucleotide comprising a nucleotide sequence of at least one of 30 contiguous nucleotides derived from a nucleotide sequence selected from the group consisting of SEQ ID NOs:1, 3, 5, 7, 9, 11, 13 and the complement of such nucleotide sequences;
 - (b) introducing the isolated polynucleotide into a plant cell;

(c) measuring the level of a polypeptide in the plant cell containing the polynucleotide; and

(d) determining the level of polypeptide in the plant cell to provide a positive selection means.

5 12. The method of Claim 11 wherein the isolated polynucleotide consists of a nucleotide sequence selected from the group consisting of SEQ ID NOs:1, 3, 5, 7, 9, 11, and 13 that codes for the polypeptide selected from the group consisting of SEQ ID NOs:2, 4, 6, 8, 10, 12, and 14.

10 13. A method of selecting an isolated polynucleotide that affects the level of expression of a plant lecithin:cholesterol acyltransferases polypeptide in a plant cell, the method comprising the steps of:

(a) constructing an isolated polynucleotide of Claim 1;

(b) introducing the isolated polynucleotide into a plant cell;

15 (c) measuring the level of polypeptide in the plant cell containing the polynucleotide; and

(d) determining the level of polypeptide in the plant cell to provide a positive selection means.

14. A method of obtaining a nucleic acid fragment encoding a plant lecithin:cholesterol acyltransferases polypeptide comprising the steps of:

20 (a) synthesizing an oligonucleotide primer comprising a nucleotide sequence of at least one of 30 contiguous nucleotides derived from a nucleotide sequence selected from the group consisting of SEQ ID NOs:1, 3, 5, 7, 9, 11, 13 and the complement of such nucleotide sequences; and

(b) amplifying a nucleic acid sequence using the oligonucleotide primer.

25 15. A method of obtaining a nucleic acid fragment encoding a plant lecithin:cholesterol acyltransferases polypeptide comprising the steps of:

(a) probing a cDNA or genomic library with an isolated polynucleotide comprising a nucleotide sequence of at least one of 30 contiguous nucleotides derived from a nucleotide sequence selected from the group consisting of SEQ ID NOs:1, 3, 5, 7, 9, 11, 13 and the complement of such nucleotide sequences;

30 (b) identifying a DNA clone that hybridizes with the isolated polynucleotide;

(c) isolating the identified DNA clone; and

(d) sequencing the cDNA or genomic fragment that comprises the isolated DNA clone.

35 16. A method for evaluating at least one compound for its ability to inhibit the activity of a plant lecithin:cholesterol acyltransferases, the method comprising the steps of:

(a) transforming a host cell with a chimeric gene comprising a nucleic acid fragment encoding a plant lecithin:cholesterol acyltransferases, operably linked to suitable regulatory sequences;

5 (b) growing the transformed host cell under conditions that are suitable for expression of the chimeric gene wherein expression of the chimeric gene results in production of the plant lecithin:cholesterol acyltransferases encoded by the operably linked nucleic acid fragment in the transformed host cell;

(c) optionally purifying the plant lecithin:cholesterol acyltransferases expressed by the transformed host cell;

10 (d) treating the plant lecithin:cholesterol acyltransferases with a compound to be tested; and

(e) determining the activity of the plant lecithin:cholesterol acyltransferases that has been treated with a test compound thereby selecting compounds with potential for inhibitory activity.

15 17. An isolated polynucleotide comprising at least one of 30 contiguous nucleotides derived from a nucleotide sequence selected from the group consisting of SEQ ID NOs:1, 3, 5, 7, 9, 11, 13, and the complement of such sequences.

18. A method of positive selection comprising:

(a) transforming a plant cell with a chimeric gene of Claim 5; and

20 (b) growing the plant cell to allow expression of the polynucleotide to alter the concentration of phytosterol in the cell to provide a positive selection means.

FIGURE 1

Arabidopsis_ (gi 3935185)	corn_ (SEQ ID NO:2)	corn_ (SEQ ID NO:4)	corn_ (SEQ ID NO:6)	soybean_ (SEQ ID NO:8)	Arabidopsis_ (gi 3935185)	corn_ (SEQ ID NO:2)	corn_ (SEQ ID NO:4)	corn_ (SEQ ID NO:6)	soybean_ (SEQ ID NO:8)	Arabidopsis_ (gi 3935185)	corn_ (SEQ ID NO:2)	corn_ (SEQ ID NO:4)	corn_ (SEQ ID NO:6)	soybean_ (SEQ ID NO:8)	Arabidopsis_ (gi 3935185)	corn_ (SEQ ID NO:2)	corn_ (SEQ ID NO:4)	corn_ (SEQ ID NO:6)	soybean_ (SEQ ID NO:8)	
<p> MKK-----I-SSHYSVVIAILVVVTMTSMCQAVGSN-----VYPLILVPGNGGNQLEVR MAR-IPQVLAPLLLLLLPAGLR--ELMIDRRPLPKRCRREVLLHPLVLPGLTCSELDAR MVHDMAS-----CSRGGTIVLSKFASSTRAPKQ-----LPPVVVPPGYATNELDAR MASSLLQQLLSLLLLLPSPLRLREHLSGNHAVSAN-----NFHPIFLVAGVSCSDLEAR MKKEQEEGL-KIEVATLTVTVVVVVMSLLCTCGASN-----LDPLILIPGNGGNQLEVR </p>					<p> LDREYKPSVWCSSWLPIHKKSGGWFRWFDAAVLLSPFT-RCFSDRMMLYYDPDLDDY LTDAYRPFRAACDE-----GEGLVRLWTNCSDLPAHHYVRCFMEQMALVYDPVANDY LTELYHPSSPRCA-----HKGKGWFRLYLNYTALEDAADVRCFAEQMATAYDAASDDY LTEEYRPSVPHCGA-----MKGKGWFGWLKNSSELLSRDYVQCFFEQMSLVYDPAINEY LTNQYKPSTFICESW-YPLIKKKNGWFRWFDSSVILAPFT-QCFAERMTLHYHQEELDDY </p>					<p> QNAPGVQTRVPHFHFGSTKSLLYLDPRLRDATSYMEHLVKALEKKCGYVNDQTIL RNLPGVETRVRFNFGSSRGF-QKNPEHTTWSWCFEVLRLNELARA-GYRDGDTLFG RNAQGVETRVFPFFGGSTRAFRYPDPDRNF-SYMDKFVSRLERL-AYRDGENLFG RNLAGVETRVFPNFGSTRAFSHKNPLKSD--WCLGKLRAALEDG-MGYRDGDTMFG FNTPGVETRVFPHFGSTNSLLYLNPRLKHITGYMAPLVDSLQKL-GYADGETLFG </p>					<p> GLAASGHPSRVASQFLQDLKQLVEKTSSENEGKPVILL APPVPGQPSRSSPATSVGWPSLVEDASRKNRGRKVVILFG AVAPPGHPSRVADAFFGLRRLRLVERASRANGGPPVTIVA APPSPGQTSSEVYSRYFKELMELVEAASERTR-KKAVILG GLAAEGHPSQVGSKFLKDLKNLIEEASNSNNGKPVILL </p>					<p> RKYIKHFVALAAPW-GGTISQMKTFASGNTLG-VPLVNPL--LVRRHQRTSESQWLLPS DRYIKHLFLVAPVPAEGFVKPLQYFVSGSNLMYVPTVSSLEPAFRPMWRTFESSLVNFPS RREVRRFVPVAAAPW-GGVVLGMLTIVAGNNLG-LPFVDPL--ALKGEYRSLSQSSWLPLPN REHIERLVLVAPTLPGGFLEPVRNFASGTDILYVPATTPLAT--RAMWRSFESAIVNFPS KKFIKHFIALSAPW-GGAIDEMYTFASGNTLG-VPLVDPL--LVRDEQRSSESNLWLLPN </p>



FIGURE 1

Arabidopsis_ (gi 3935185)	TKVFHDKPLVVTPOVNYTA--YEMDRFFADIGFSQGVVPYKTRVLPLTEELMTPGVPV		
corn_ (SEQ ID NO:2)	PAVFGR--RPLVVTARRNYS--YDLELLVAVGYGAGVEFRRRAVPKMSYFQAPMVPT		
corn_ (SEQ ID NO:4)	PNAFRA-GQPLVTTTRSRITYTA--HDMADFLDAIGLGAIVPYQSRVLPLFRELPSPRPV		
corn_ (SEQ ID NO:6)	PAVFGRQLQAPLVVTRERNYSASAHDMERFLAAGSGEAAEPFRRRAVPKMGSAAPMVPM		
soybean_ (SEQ ID NO:8)	PKIF-GPQKPIVITPIRPYSA--HDMVDFLKDIGFPEGVYPYETRIPLIGNIKAPQVPI		
Arabidopsis_ (gi 3935185)	TCIY	KQPEIK-	ASLAAL-----KVDS-LN
corn_ (SEQ ID NO:2)	TCMN	ATPEIV-	VSMLAFDEKMRRQPEQNKVYK
corn_ (SEQ ID NO:4)	ACVV	VTPMMV-	VSL LAVDP-AWRLPTA-Y-FR
corn_ (SEQ ID NO:6)	TYIS	AAPEVAA	ISVLA FEKEMRRQPEQKKQFK
soybean_ (SEQ ID NO:8)	TCIM	ERPEIS-	VSL LALQS-LWKEEKNQY-LK
Arabidopsis_ (gi 3935185)	TVEIDGVSHTSILKDEIALKEIMKQISIINY--ELANVNAVNE		
corn_ (SEQ ID NO:2)	SIKIRGAQHGTIVTDDTALKRVMHEILEANR-----S		
corn_ (SEQ ID NO:4)	MLKVRNVSHGTGLFVDDAALAVIISAI-----LRPN		
corn_ (SEQ ID NO:6)	SIKINKAQHSTIVTDDFALHRVIEI VEANNQK-----IP\$		
soybean_ (SEQ ID NO:8)	VVKIDGVSHTSILKDEVALNEIVGEITSINSHAEGLSLNLFSG		

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1. 10/10/10




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INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 99/28586

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/54 C12N15/82 C12N9/10 C12Q1/02 C12Q1/48
C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SASAKI T. ET AL.: "Rice EST AC D46513" EBI DATABASE, 9 March 1995 (1995-03-09), XP002139389 the whole document	1-9
X	SHINN P. ET AL.: "Genomic Arabidopsis sequence AC AC004557; Q9ZW42" EBI DATABASE, 15 April 1998 (1998-04-15), XP002139390 the whole document	1-10, 16

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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 "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
 "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
 "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
 "&" document member of the same patent family

Date of the actual completion of the international search

5 June 2000

Date of mailing of the international search report

26/06/2000

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Kania, T



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2

3

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/28586

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>WARDEN C. ET AL.: "Tissue-specific expression, developmental regulation, and chromosomal mapping of the lecithin:cholesterol acyltransferase gene" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 264, 1989, pages 21573-21581, XP002139391 the whole document</p> <p style="text-align: center;">---</p>	1-18
A	<p>MURATA Y. ET AL.: "Cloning of rabbit LCAT cDNA: increase in LCAT mRNA abundance in the liver of cholesterol-fed rabbits" JOURNAL OF LIPID RESEARCH, vol. 37, 1996, pages 1616-1622, XP000913765 the whole document</p> <p style="text-align: center;">---</p>	1-18
A	<p>WO 98 46767 A (TANIYAMA YOSHIO ; TAKEDA CHEMICAL INDUSTRIES LTD (JP)) 22 October 1998 (1998-10-22) see the whole document; esp. p.6 l.14 ff.</p> <p style="text-align: center;">---</p>	1-18
P,X	<p>WALBOT V. ET AL.: "Maize EST, AC AW147120" EBI DATABASE, 4 November 1999 (1999-11-04), XP002139392 the whole document</p> <p style="text-align: center;">-----</p>	1-9

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 99/28586

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9846767 A	22-10-1998	AU 6748198 A	11-11-1998
		EP 0973914 A	26-01-2000
		JP 11269199 A	05-10-1999
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CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
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DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						



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MAR 09 2001

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

By fax and post

To:

SCHAEFFER, Andrew L.
E. I. du Pont de Nemours and Company
Legal Patent Records Center
1007 Market Street
Wilmington, DE 19898
ETATS-UNIS D'AMERIQUE

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MAR 07 2001

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THE INTERNATIONAL PRELIMINARY
EXAMINATION REPORT

(PCT Rule 71.1)

FAX: 302 773 0164

Date of mailing
(day/month/year)

28.02.2001

Applicant's or agent's file reference
BB1262

IMPORTANT NOTIFICATION

International application No.
PCT/US99/28586International filing date (day/month/year)
02/12/1999Priority date (day/month/year)
03/12/1998

Applicant

E.I DU PONT DE NEMOURS AND COMPANY ET AL

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.
2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.
3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. REMINDER

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/

 European Patent Office
D-80298 Munich
T I. +49 89 2399 - 0 Tx: 523656 epmu d
Fax: +49 89 2399 - 4465

Authorized officer

Emslander, S

Tel. +49 89 2399-8718

KEY NOTED



03 JE 2001



PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)



Applicant's or agent's file reference BB1262		FOR FURTHER ACTION See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/US99/28586	International filing date (day/month/year) 02/12/1999	Priority date (day/month/year) 03/12/1998	
International Patent Classification (IPC) or national classification and IPC C12N15/54			
Applicant E.I DU PONT DE NEMOURS AND COMPANY ET AL			

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.
2. This REPORT consists of a total of 5 sheets, including this cover sheet.
 - ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☒ Certain observations on the international application

Date of submission of the demand 26/06/2000	Date of completion of this report 28.02.2001
Name and mailing address of the international preliminary examining authority:  European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Stolz, B Telephone No. +49 89 2399 8416 



**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT**

International application No. PCT/US99/28586

I. Basis of the report

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*:

Description, pages:

1-17,19-27 as originally filed
18 filed with the demand

Claims, No.:

1-18 as originally filed

Drawings, sheets:

1/2,2/2 as originally filed

Sequence listing part of the description, pages:

1-15, as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
☐ the language of publication of the international application (under Rule 48.3(b)).
☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☒ contained in the international application in written form.
☒ filed together with the international application in computer readable form.
☐ furnished subsequently to this Authority in written form.
☐ furnished subsequently to this Authority in computer readable form.
☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
☐ The statement that the information recorded in computer readable form is identical to the written sequence

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT**

International application No. PCT/US99/28586

listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	5-7, 9, 11-16, 18
	No:	Claims	1-4, 8, 10, 17
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-18
Industrial applicability (IA)	Yes:	Claims	1-18
	No:	Claims	

2. Citations and explanations
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

1. Reasoned statement

1.1. The application discloses ESTs from corn and soybean putatively encoding Lecithin:cholesterol acyltransferase (LCAT). The claimed function has been inferred from sequence homology of the most likely reading frame with known animal LCAT. The claimed sequences have not been expressed. Thus, there is no evidence that the claimed sequences indeed encode peptides having LCAT activity. Nevertheless, applicants claim not only the nucleotide sequences but also the deducible peptides and their conceivable uses.

1.2. Novelty (Art. 33(2) PCT)

As a preliminary remark, it is noted that the priority document only discloses Seq. ID nos. 1 to 6. Seq. ID nos. 7 to 14 are for the first time disclosed at the international filing date.

Claim 1 refers to polynucleotides comprising nucleotide sequences encoding peptides of at least 50 amino acids having at least 60% identity with the peptides of Seq. ID nos. 2 to 14. Such a nucleotide sequence is disclosed in document XP-002139390 (D1, AC 004557, *A. thaliana*). D1 has last been updated on 02.12.1998, which is before the priority date of the instant application. The nucleotide sequence disclosed in D1 is 64% identical in a 743 nucleotide overlap with Seq. ID 5, and encodes a protein having 64% sequence identity in a 369 amino acid overlap. The protein is identified as a putative LCAT. In consequence, D1 affects also the novelty of claims 2 to 4, 8, 10, and 17. A further nucleotide sequence falling under claim 1 is described in XP-002139389 (D2). The sequence is 73% identical in a 324 nucleotide overlap. As far as Seq. ID no. 7 is concerned, its novelty is also affected by XP-002139392, cited as a P-document.

1.3. Inventive step (Art. 33(3) PCT)

All claims lack inventive step in view of D1. D1 disclosed a reading frame from *A. thaliana* encoding a putative LCAT. The uses of the disclosed sequence according to any of the remaining claims are immediately obvious to the person of skill.

Furthermore, the specifically defined sequences of Seq. ID nos. 1 to 14 are not inventive. The claimed sequences differ from those of D1 only by structural features. They have not been expressed and functionally characterised. All functional properties of the new sequences have been inferred from their homology to known animal LCAT. With regard to functional properties, the instant application is therefore on the same speculative level as D1. In view of D1, the contribution of the present application is then the mere provision of further molecules showing homology to the known LCATs. Finding further molecules showing such homology does however not require inventive skills. The person of skill could have used any of the known LCAT sequences to screen EST databases or to devise probes for screening cDNA or genomic libraries. From D1 it was apparent that plant DNA could also be screened. Each of the so identified molecules would have presented an equally suitable contribution to the art. Applicants selected the molecules defined by Seq. ID nos. 1 to 14 from this larger group of possible contributions. Such a selection from a larger group is considered to be arbitrary. However, an arbitrary selection cannot involve an inventive step because, in order to be patentable, the selection must not be arbitrary but must be justified by the technical purpose, e.g. by a hitherto unknown or unexpected effect which is caused by those structural features distinguishing the claimed compounds from the numerous other ones. The application lacks any experimental data in that respect and therefore cannot show any such effect. As a consequence, all claims are considered to lack inventive step, even when restricted to the specific molecules of Seq. ID nos. 1 to 14.

2. Certain observations

- 2.1.** There is a discrepancy between the description of Fig. 1 (e.g. p.3) and the contents of Fig. 1. While the description refers to Seq. ID nos. 8, 10, 12, and 14, the figure displays Seq. ID nos. 2, 4, 6, and 8.

